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The purpose of this research proposal is to develop an autologous breast cancer vaccine whose immunogenicity we hypothesize will be significantly enhanced by the ectopic expression of the human T cell costimulatory molecule, B7-1. To accomplish this goal, several experiments were conducted that demonstrated a) the feasibility of short-term culture of fresh, human breast cancer cells, b) the efficiency of transduction of these cells with the adenoviral expression vector, Ad. hB7 resulting in expression of the B7-1 protein on the cell surface, and c) the capability of the ectopic expression of B7-1 to costimulate human T cells. In preparation for a clinical trial, toxicology studies in mice of the murine B7 adenoviral vector were performed as required by the FDA. The toxicity study in 120 mice of escalating doses of the Ad.mB7-1 vector has been completed. Following the compilation of all the pathologic and immunologic results, the IND will be submitted to the FDA in December, 1997. Accrual of patients onto the phase I clinical trial to determine the toxicity and immunologic effects of an autologous B7-modified breast cancer vaccine (Task 3 and 4) is anticipated to begin in the first quarter of 1998.

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PI - Signature 10.28.97 Date

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I. Introduction

The purpose of this research is to develop an autologous breast cancer vaccine whose immunogenicity we hypothesize will be significantly enhanced by genetic modification with an adenoviral expression vector for the T cell co-stimulatory molecule, B7-1.

The technical objectives of this research as outlined in the original proposal are to:

1. Develop methods to isolate highly purified populations of breast cancer cells from tissue specimens of locally recurrent or metastatic sites obtained from patients with advanced, refractory breast cancer.

2. Study and optimize the efficiency and longevity of B7 expression in breast cancer cells transduced with adenoviral vectors containing the human B7 cDNA.

3. Conduct a Phase I clinical trial assessing the toxicity of autologous B7-transduced irradiated breast cancer cells used as a vaccine to enhance the immune response to the tumor.

4. Perform in vitro and in vivo immunologic monitoring studies on enrolled patients to assess the development of an anti-tumor immune response.

II. Body

Brief history of the grant

This research project proposal was submitted by the University of Michigan to the US Army Medical Research and Materiel Command in November 1993. It was approved in May 1994 and funding started in October 1994. The principal investigator was John W. Smith II, M.D., then an Associate Professor in the Department of Internal Medicine at the University of Michigan involved in clinical research in breast cancer and immunotherapy. Associate investigators included Laurence A. Turka, M.D., then an Associate Professor in the Department of Internal Medicine, Director of the University of Michigan Cancer Center Tumor Immunology Program and recognized expert in T cell costimulation pathways, Stephen P. Ethier, Ph.D., Assistant Professor in the Department of Radiation Oncology and leader in the field of breast cancer cell biology, and Alfed E. Chang, M.D., Professor of Surgery and Chief, Division of Surgical Oncology, Associate Director for Clinical Affairs of the University of Michigan Cancer Center and a established authority in the field of cancer immunotherapy. Included in the original grant was a subcontract with James M. Wilson, M.D., Ph.D., Director of the Institute of Human Gene Therapy, University of Pennsylvania and his colleague, Stephen L. Eck, M.D., Ph.D. More details regarding the investigators qualifications and budget justifications are found on pages 22-36 of the original research project proposal.

In the summer of 1995, Dr. Turka moved to the University of Pennsylvania but was retained as a consultant through a subcontract to the University of Pennsylvania. In September, 1996, Dr. Smith moved to Portland, OR where he serves as Chief, Clinical Research, Earle A. Chiles Research Institute, Providence Portland Medical Center. Because the Army Medical Research and Materiel Command considers that the research project grants are made to institutions and not individuals, a new Principal Investigator at the University of Michigan was named:

Laurence H. Baker, D.O.
Professor of Medicine and Associate Chief,
Division of Hematology/Oncology,
Department of Internal Medicine
University of Michigan School of Medicine
Director for Clinical Research and Deputy Director
University of Michigan Comprehensive Cancer Center

The University of Michigan has established a subcontract with the Earle A. Chiles Research Institute to allow Dr. Smith to continue to help coordinate the research grant especially with the performance of tasks 3 and 4 in the statement of work, i.e. to conduct a Phase I clinical trial of B7 transduced autologous breast cancer cells as a vaccine and to perform immunological monitoring studies on the patients participating in

the trial. The joint enrollment of patients at the University of Michigan and the Providence Medical Center will be advantageous because the patient population (i.e. breast cancer patients with an easily obtainable source of autologous tumor) is relatively rare. Patient accrual will be improved by having both centers involved. Because the start of the clinical trial has been delayed by difficulty in manufacturing the adenoviral vector, there is less time to conduct the study and it would be extremely difficult for a single institution to complete the trial within the remaining time on the grant. The Earle A. Chiles Research Institute, Portland Providence Medical Center was the first institution to conduct a gene therapy protocol in the state of Oregon. Currently, there are two active gene therapy studies at that institution, including a protocol for breast cancer patients, funded by the NIH, that is similar to the present Army grant. Their proposal differs because it uses an HLA-A2 matched, allogeneic human breast cancer cell line (MDA-MB-231) instead of using autologous tumor, but both are transducing the breast cancer cells with B7 (CD80) to make the tumor immunogenic. Therefore, Dr. Smith and his colleagues at the Earle A. Chiles Research Institute are completely capable and eminently qualified to conduct the clinical protocol and immunological monitoring as described in the statement of work. Dr. Smith will be in frequent contact with his collaborators and in addition, will make two trips per year to the University of Michigan for on site meetings. Additional details about the changes in the principal investigator and the subcontract with the Earle A. Chiles Research Institute can be found in the appended documents.

Task 1

Task 1 in the Statement of Work is to develop methods to isolate highly purified populations of breast cancer cells from tissue specimens of locally recurrent or metastatic sites obtained from patients with advanced, refractory breast cancer. The work is being conducted in the laboratory of Stephen P. Ethier, Ph.D. at the University of Michigan. The methods that were proposed in the original grant are described in detail below.

> *Specific Aim #1. Selective isolation of human breast cancer cells from primary tumors and metastases using monoclonal antibody-conjugated magnetic beads.*
>
> **Rationale:** In order to isolate a highly pure population of human breast cancer cells that are suitable for infection with adenoviral expression vectors it is necessary to prepare a viable single cell suspension of cells from breast cancer specimens. In our previous studies with primary and metastatic human breast cancer specimens we have used an enzymatic dissociation procedure to prepare breast cancer cells for cell culture experiments. For cell culture applications, generation of cell suspensions that consist of multi-cell aggregates of breast cancer cells and normal cells is sufficient and even advantageous in some ways. For the experiments to be performed in the present studies, the cell suspensions obtained following enzymatic dissociation of breast tissue specimens will be treated further

to prepare single cell suspensions. Single cell suspensions prepared from these specimens will be exposed to a panel of monoclonal antibodies in order to separate normal cells from malignant cells. Finally, the isolated breast cancer cells will be infected with adenoviral expression vectors, containing either a reporter gene (LacZ) for developmental studies, or the B7 gene for gene therapy experiments.

a. Preparation of single cell suspensions of human breast cancer cells.

Solid tumor specimens, either primary tumors or solid metastatic nodules, will be minced with sterile scalpels until tissue pieces are approximately 1 mm^3. The minced tissues will be suspended in Medium 199 containing Worthington type III collagenase (Worthington Chemical Co., Freehold, NJ) at a concentration of 200 units per ml, and Dispase (Boehringer-Mannheim, Indianapolis, IN) at a concentration of 1 mg per ml. Twenty mls of enzyme solution are used per gram of tissue. The tissues are incubated overnight in a 37° water bath shaking at 65 rpm. The next day, the remaining tissue clumps are mechanically dissociated by repeated pipetting of the suspension. The cells are then washed three times by centrifugation at 250 x g and re-suspended in fresh Medium 199 after each wash. This enzymatic dissociation procedure results in a mixed suspension of single cells, small aggregates and large mammary organoids. The viability of the cells in this suspension is greater than 95%. To prepare a single cell suspension from the mixed aggregate population, the cells will be washed in Ca^{++}, Mg^{++}-free, Hanks balanced salt solution (CMF-Hanks BSS) and then incubated for 4 hours in CMF-Hanks BSS containing 10 mM EDTA, at 4° with gentle rocking. The cells will be mechanically dissociated every hour during the four hour period by repeated pipetting of the cell suspension. If necessary to maintain viability of the cells during this incubation, the CMF-Hanks-EDTA solution will be supplemented with 5% fetal bovine serum that had been treated with Chelex to remove divalent cations. After the four hour incubation, single cells are separated from any remaining cellular aggregates by filtration through Nitex mesh with a 20 um pore size.

Our preliminary data indicate that collagenase/Dispase dissociation of breast tissues does not adversely effect the integrity of cell surface molecules as these aggregates are quite reactive to antibodies directed against cell surface proteins. In generation of single cell suspensions, however, it is imperative that a method be used that does not alter the peptide epitope present on the surface of breast cancer cells. For this reason, we have chosen to use chelating agents that disrupt cell to cell interactions without degrading cell surface molecules to achieve the final single cell suspensions. It is necessary to obtain single cell suspension for the final cell purification procedures as the separation methods make use of antibodies that bind to epitopes expressed on beast cancer cells and not on normal mammary epithelial cells. If cell aggregates are used in the cell isolation

procedures and if aggregates contain both normal and neoplastic cells, then the purpose of using breast cancer specific antibodies would be defeated.

b. Isolation of breast cancer cells using antibody conjugated magnetic beads.

The basic strategy for isolating breast cancer cells involves the use of magnetic beads (Dynabeads, Dynal Inc. Great Neck, NY) that have been conjugated with anti-mouse IgG antibodies. Thus, the anti-mouse antibodies on the beads can be bound to mouse monoclonal antibodies directed against cell surface epitopes to prepare a reagent that specifically binds cells expressing the epitope. Following incubation of a cell suspension with antibody coated magnetic beads, the bound cells can be separated from non-bound cells by placing the tube in magnetic particle concentrators (MPC) designed to hold microfuge tubes. The beads and bound cells adhere tightly to the wall of the MPC and the non-bound cells are aspirated from the tube. The tube is then removed from the MPC, the cells re-suspended in medium and this washing procedure is repeated three to four times. With this method, we have separated mixed cell populations with greater than 99% efficiency using antibodies against the erbB-2 protein and antibodies against breast epithelial mucins. The cells isolated in this way have been seeded into culture and exhibit high viability as indicated by their ability to attach in culture and proliferate.

To coat magnetic beads with mouse monoclonal antibodies, 1×10^8 anti-mouse IgG Dynabeads are suspended in 1 ml of CMF-Hanks BSS and incubated with 1 μg of mouse monoclonal antibody with rocking at room temperature for two hours. Following the incubation, the beads are washed extensively with CMF-Hanks BSS by adhering the beads to the tube wall using the MPC, aspirating the medium, re-suspending the beads in 1 ml of fresh medium and incubating with rocking for 30 minutes. This washing procedure is repeated three times. After the last wash, the beads are suspended in CMF-Hanks BSS at a concentration of 10^8 beads per ml.

To isolate cells using antibody conjugated magnetic beads, 2×10^7 magnetic beads are added to a 1 ml aliquot of a cell suspension of 1×10^7 cells and incubated with rocking at room temperature for two hours. Next, the cells bound to the beads are washed three times to separate them from non-bound cells. If necessary, the beads can be removed from the purified cells either by trypsinization or by incubation with the peptide epitope that was used to generate the primary antibody. The cells isolated in this way can be used to initiate cell cultures of breast cancer cells or can be infected with adenoviral expression vectors. For the experiments to be carried out in this project, we will employ magnetic beads coated with three different antibodies. The first antibody, Sm-3, was generated against the core peptide of breast epithelial mucins. As discussed earlier, altered glycosylation of mucins that occurs in greater than 90% of breast cancer cells reveals the peptide epitope that is masked in normal cells by glycosylation. Thus,

the Sm-3 antibody coated beads will be the primary antibody for isolating human breast cancer cells from primary tumor specimens that contain both normal and neoplastic mammary epithelial cells. The Sm-3 antibody was obtained from Dr. Joy Burchell, Imperial Cancer Research Fund, London, UK. A second antibody, Mc-5, recognizes breast epithelial mucins expressed on virtually all breast cancer cells. This antibody also binds to normal mammary epithelial cells making it less useful for primary tumor specimens. However, magnetic beads coated with this antibody have been used in our laboratory to isolate breast cancer cells from metastatic lymph nodes and pleural effusion metastasis. This antibody was obtained from Dr. J. Peterson, Cancer Research Fund of Contra Costa, Walnut Creek, CA. Finally, a third antibody, Tab-254, binds to the extra-cellular domain of the erbB-2 protein. Magnetic beads conjugated with this antibody are useful in isolating breast cancer cells from primary or metastatic sites that overexpress the erbB-2 protein as a result of amplification of the c-erbB-2 (Her-2/neu) gene, which occurs in approximately 30% of breast cancer cases. A panel of Tab antibodies against the erbB-2 protein have been obtained from Dr. Beatrice Langton, Berlex Biosciences, Richmond, CA.

During the past year, Dr. Stephen Ethier's developed improved methods for the isolation and cultivation of primary human breast cancer cells in vitro. His laboratory has now isolated and characterized ten new human breast cancer cell lines. Four of these cell lines are from primary tumors, one was isolated from a metastatic lymph node, one is from a skin metastasis, one was from a recurrent chest wall lesion and the remaining cell lines are from pleural effusion metastasis or malignant ascites. The cell lines express the range of oncogene changes known to occur in human breast cancer including; erbB-2 amplification and overexpression, overexpression of epidermal growth factor receptor, amplification of the FGFR 1 and 2 genes, mutations in P53 and alterations in pRB protein expression. A summary of the molecular characteristics of all the human breast cancer cell lines that have been isolated and cultivated by Dr. Ethier's lab is found in Table 1 and more details can be found in references 3-7.

These lines represent the kind of range of human breast cancer cells that will need to be used in gene therapy studies. Furthermore, the isolation of these lines is indicative of the progress that his lab has made in developing methods and conditions for the routine isolation of human breast cancer cells. Thus, successful short term culture of human breast cancer cells is now possible for the majority of patient samples, and long-term cell culture of these breast cancer cells is possible with roughly 20% to 30% of specimens.

Dr. Stephen Ethier's laboratory has performed some experiments to purify breast cancer cells that were only marginally successful. They used the Sm-3 antibody, which is reported to be luminal cell specific, attached to magnetic beads to purify cells. The overall methodology worked well, but the antibody was not specific enough and many cancer cells were not purified using this approach. They are currently doing more experiments

with magnetic beads using antibodies to a cell surface marker called CALLA-1 and to Muc-1 and expect that these will be more useful in purifying breast cancer cells. They are also using a microgravity cell generator to selectively isolate breast cancer cells. The differential ability of breast cancer cells to survive and grow in suspension is the basis of this method and thus far they have found that a 6 to 10 day culture in suspension results in death of the majority of normal cells and the survival of the breast cancer cells. It is expected that in future experiments, the antibody-magnetic bead approaches will be combined with the microgravity cell generator to obtain nearly pure populations of breast cancer cells.

Task 2

Task 2 in the Statement of Work is to study and optimize the efficiency of and longevity of B7 expression in breast cancer cells transduced with adenoviral vectors containing the human B7 cDNA. The work is being conducted in the laboratory of Stephen P. Ethier, Ph.D. at the University of Michigan. The methods that were proposed in the original grant are described in detail below.

> *Specific Aim #2. Study and optimize the efficiency and longevity of B7 expression in breast cancer cells transduced with adenoviral vectors containing the human B7 cDNA.*
>
> **Rationale:** Prior to the use of transduced breast cancer cells as immunotherapy the methodologies to transduce the maximum number of cells with an adenoviral vector and to verify that large numbers of cells express the vector-encoded human gene for at least several days must be developed. In this specific aim, we will perform a series of experiments aimed at optimizing methods for the infection of purified human breast cancer cells with adenoviral expression vectors and for optimizing the expression of a transgene within these vectors. Experiments will also be performed to determine the immunogenicity of human breast cancer cells that express the B7 gene following infection with appropriate adenoviral expression vectors.
>
> *a. Infection of purified human breast cancer cells with adenoviral expression vectors.*
> To perform the optimization experiments, an adenoviral vector containing a reporter gene (LacZ) as the transgene will be used. In preliminary studies with early-passage breast cancer cell lines developed in our laboratory, we have found that overnight exposure of these cells to these adenoviral vectors results in expression of the LacZ transgene in greater than 80% of the cells. To optimize infection of purified human breast cancer cells with the adeno-LacZ virus, aliquots of 10^6 human breast cancer cells, purified using methods described above, will be

incubated in suspension with adeno-LacZ virus for 24 hours with gentle agitation. Multiplicity's of infection ranging from 10^2 to 10^4 pfu's per cell will be tested in these experiments. Following infection, cells will be seeded into culture using media that we have developed for human breast cancer cell growth, and the cells will be assessed for LacZ activity at 24 hours, 3, 7, 10 and 14 days after infection. This experiment will be carried out with cells from at least 10 separate breast cancer patients. In these experiments, we will determine the optimum multiplicity of infection and the duration of the transgene expression in purified human breast cancer cells infected with adenoviral vectors immediately after their isolation.

b. Infection of purified human breast cancer cells with B7-adenoviral vectors.

Experiments will then be performed to transduce human breast cancer cells with the human B7 gene using adenoviral vectors developed by James Wilson and Steve Eck (see letter of consultancy). For these experiments the conditions shown to yield optimal transduction of the LacZ reported gene will be used for B7. Expression of B7 protein on the surface of the human breast cancer cells will be assessed by flow cytometry using CTLA4Ig or commercially available mouse anti-human B7 mAb. We will also verify that the cells are capable of supporting B7-mediated responses such as providing co-stimulatory signals (as assessed by proliferation and IL-2 gene expression) for autologous T lymphocytes activated with phorbol ester, bacterial superantigens, or PHA (methods as outlined under specific aim #4). Each of these is an accessory cell dependent stimulus, however we and others have shown that purified T cells can respond to these stimuli in the presence of B7-transfected CHO cells. Thus we will use B7-transfected CHO cells as a positive control in these studies. This also will allow us to compare the relative co-stimulatory abilities of $B7^+$ CHO cells and autologous $B7^+$ breast cancer cells. Since in the design of our phase I study (specific aim #3) the cells will be irradiated with 5000 cGy prior to injection into patients, we will also verify in these studies that the cells retain co-stimulatory capacity after irradiation at this dosage.

In previous reports, we have indicated that human breast cancer cells infected with an adenoviral expression vector containing that LacZ gene, express the transgene at very high frequencies (approximately 90%) and maintain expression of the transgene for several weeks following infection. More recently, Dr. Ethier's lab has performed experiments with an adenoviral expression vector containing the human B7-1 gene. This vector is essentially the same vector that will be used to prepare the autologous tumor cell vaccines for the clinical trial. The experiments performed thus far have made use of this vector (AdB7-1) and a series of human breast cancer lines that he has developed in his laboratory. There are a number of advantages of the cell lines he has developed. Since they were developed in his lab, they have all been tested in early passage, and thus are more representative of the breast cancer cell primary cultures that will eventually be used in the clinical trial. In addition, his lines come from a wide range of breast cancer

specimens, ranging from early stage primary tumors, to large inflammatory primary tumors to metastatic specimens and chest wall recurrences.

To do these experiments, cells from the human breast cancer cell lines were cultured to high density and then infected for 2 hours with the AdB7.1 vector at various multiplicity's of infection (MOI). After 48 hours, the cells were harvested from the dish using 10 mM EDTA, and incubated with the high affinity B7 binding protein CTLA4Ig. The cells were then incubated with a fluorescent secondary antibody, washed extensively and then scanned by flow cytometry.

The FACs scans shown in figure 1 illustrate the detection of B7.1 expression in the human breast cancer cell lines 48 hours after infection. Table 2 shows the data obtained from these scans and indicates the percent B7 positive cells following infection at two different MOIs. These results indicate that all of the human breast cancer cell lines studied were successfully infected and expressed the B7 protein on the cell surface. Although there was some variability from experiment to experiment, these experiments indicated that at MOIs of 10^4 particles per cell, the vast majority of breast cancer cells express high levels of the protein on the cell surface. At lower MOIs, most of the breast cancer cells did express the B7 protein, but the proportion of positive cells was lower.

These results clearly indicate that human breast cancer cells from many different patients and derived from both primary and metastatic sites are successfully infected with the AdB7.1 vector and express high levels of the protein on the cell surface. In addition, the expression of B7.1 protein following infection is independent of the growth rate of the breast cancer cell lines. Indeed, SUM-44 cells, which still have doubling times of approximately 200 hours, are as readily infected as SUM-149 cells, which grow much more rapidly. Experiments currently underway are aimed at extending these experiments to studies with cells derived directly from patient samples, and examining the influence of radiation exposure on the long-term expression of B7 protein in these cells.

<u>Task 3</u>

Task 3 in the Statement of Work is to conduct a Phase I clinical trial of B7 transfected breast cancer cells as a vaccine. Patients will be enrolled onto the clinical trial at the University of Michigan and at Portland Providence Medical Center. The detailed and complete phase I protocol was included in the original grant proposal. A summary of the methods that were proposed in the original grant are described in detail below.

> *Specific Aim #3. Conduct a Phase I clinical trial assessing the toxicity of autologous B7-transduced irradiated breast cancer cells as a vaccine to enhance the immune response to the tumor.*

Rationale: In order to test the safety of a genetically modified autologous tumor vaccine, a phase I clinical trial must be performed. Although other cancer patients have received genetically altered tumor cell vaccinations (transfected with cytokine genes), this trial would represent one of the first times a genetically altered tumor cell vaccine that contained the gene for B7 was given. Therefore, it is prudent to begin the testing in patients with advanced refractory breast cancer. This is not the optimal setting for a vaccine to work, since these patients will have a significant tumor burden and have received several chemotherapy regimens, both of which can significantly depress the immune system. Ideally, a vaccine should be utilized to treat patients with low tumor burdens such as is the case after primary definitive surgery and/or irradiation. Nevertheless, the initial toxicity and safety studies must always be performed in patients with advanced disease and limited life expectancy. We plan to administer an autologous irradiated B7-transfected breast cancer cell vaccine once to patients with advanced breast cancer and observe them closely for side effects, toxicities, any clinical anti-tumor responses, and changes in their immune response to the tumor. Cohorts of six patients each will receive one of 3 dose levels (numbers of transfected breast cancer cells transfected) in an escalating fashion as toxicity permits. Assuming toxicity is not severe, future clinical trials could consider a vaccination schedule with several planned vaccinations, as well as the addition of adjuvants (if necessary) or systemic immune stimulating agents such as IL-2 following the vaccine. Future studies could also incorporate the harvest of regional lymph nodes and expansion of TILs for adoptive immunotherapy. The details of the proposed clinical trial are described in the model protocol that is in Appendix F. The key features will be delineated below. We recognize that certain technical details are still in the developmental phase. These details will be incorporated into the final version of the protocol prior to submission to the IRB, RAC and FDA. We also wish to emphasize that the appropriate animal toxicity studies will be performed prior to the initiation of the clinical trial by our collaborators Drs. Wilson and Eck (at the University of Pennsylvania) using the type of B7-expression vectors that would be used in our human studies. These toxicity experiments will include intravenous injection of the B7 vector, as well as deliberate transduction of a variety of non-malignant cells such as hepatocytes, fibroblasts and keratinocytes. The studies will assess the animals for adverse effects including the induction of unwanted autoimmune responses.

Phase I Study Objectives.

1. To determine the toxicity of subcutaneously administered irradiated autologous breast cancer cells that have been transfected with the human gene for B7 in patients with advanced or metastatic breast cancer.
2. To determine the maximum number of transfected breast cancer cells that can be safely given to these patients.
3. To determine if the vaccination results in an immune response and to characterize that immune response.

4. To observe patients for any antitumor responses.

Eligibility Requirements.

Patients must have advanced breast cancer that has failed to respond to at least two standard chemotherapy regimens used in the metastatic setting and who are considered unlikely to benefit from further salvage chemotherapy regimens or hormonal regimens. They must also have a source of autologous tumor that can be easily harvested. This includes patients with subcutaneous or cutaneous metastases, patients with easily excisable lymph nodes containing metastatic tumor, and patients with malignant pleural effusions or ascites. Patients must have a good performance status and a life expectancy of at least three months. Patients must be at least 18 years old. There is no exclusion for sex or ethnic background. Patients must have evaluable or measurable disease in addition to the disease that will be surgically removed for the purposes of formulating the autologous vaccine. Adequate baseline organ function will be required. In addition, patients must not be anergic to standard recall antigens. Patients may not have received prior antitumor vaccines or immunotherapy. Patients will be excluded if they have any autoimmune diseases, evidence of HIV infection or AIDS, active infection, bleeding, pregnancy, or lactation, or any significant uncontrolled medical or pyschiatric illness. Patients who require corticosteroids or anticoagulation are ineligible.

Study Design.

Patients will undergo surgical removal of metastatic disease under local anesthesia in order to provide autologous tumor cells that can be transfected with the human B7 gene. A section of the removed tumor will be sent to surgical pathology for pathologic diagnosis. The remainder of the specimen will go to the laboratory to prepare B7-transfected autologous tumor, for immunologic assays, and for cryopreservation. (For details on the purification and transfection of the breast cancer cells see the technical methods section for specific aim 1 and 2). After the autologous breast cancer cells have been transfected with B7 they will be irradiated with 5000 cGy, a dose of radiation that renders them nontumorigenic but allows them to remain metabolically active. They will then be injected intradermally into the thigh approximately 10 cm below the inguinal lymph nodes and the injection site will be marked with an ink tatoo for future biopsy. The injections will be administered in the Clinical Research Center, University of Michigan Hospital and the patients will remain in the hospital over night. Cohorts of six patients each will be treated with escalating doses of autologous irradiated B7 transfected breast cancer cells according to the following scheme: a): 10^6 cells, b): 10^7 cells, c): 10^8 cells (for technical reasons, 10^8 cells is the likely maximum number of cells that could be obtained from these patients). Individual patients will receive one dose level of cells (i.e. there is no intrapatient dose escalation). Only one vaccination is planned for each patient unless the patient demonstrates clinical benefit from the treatment, whereupon the patient can receive

additional monthly vaccinations as long as such benefit persists. Each patient will be observed for at least three weeks at a given level of cell injection before the patients are permitted to enroll on the next higher dose level of cells. If one or fewer patients experience dose-limiting toxicity at a given number of cells injected, escalation will be permitted to continue to the next level. If two or more patients sustain dose-limiting toxicity, then that level of cells will be determined as the dose-limiting number of cells and the dose level of cells below that will be defined as the maximum tolerable dose of cells to be injected. It is possible that at the maximum dose of B7-transfected cells dose-limiting toxicity will not be observed.

Once escalation is completed, a separate cohort of six patients will be treated with both B7-transfected autologous irradiated breast cancer cells and vector only-transfected autologous irradiated tumor cells. One injection will be placed in the left thigh and one in the right thigh at the same time. The purpose of treating this cohort of patients is to compare the immunologic response at the vaccination site and in the draining lymph nodes from one leg to the other. This will help determine if B7 transfection enhances the immune response above that which is seen with transfection of vector alone.

Study endpoints.

TOXICITY - Patients will be closely followed and observed for the development of any clinical side effects from the treatment. Toxicity will be graded according to the Cancer Treatment Evaluation Program toxicity scale. The major toxicity that is anticipated is local redness, swelling, pain, and increased warmth at the injection site. Patients will be monitored for the development of clinical symptoms suggesting autoimmune disease or allergic reactions. Changes in laboratory parameters (complete blood count, chemistry panel, coagulation studies, urinalysis, as well as tests for the development of autoimmune disease [ANA, RF, CH50, anti-DNA abs, T4, TSH]) will be assessed two and four weeks after vaccination and thereafter once/month. In addition, sera and peripheral blood mononuclear cells will be obtained for archival purposes according to the current safety monitoring guidelines by the Center for Biologics Evaluation and Research (presently, once/month on treatment and every three months thereafter).

IMMUNE RESPONSE - A biopsy of the vaccination site along with surgical removal of one to three draining inguinal lymph nodes will be performed two weeks after the vaccination. Peripheral blood will be obtained at two weeks and 4 weeks and then once/month. DTH skin testing will be performed monthly. The details of the immunologic monitoring are extensively described in the methods section for specific aim #4.

ANTITUMOR RESPONSE - Four weeks after vaccination, the patients will undergo reevaluation to determine if their disease has responded or progressed using standard response criteria. Patients whose disease has not worsened or has regressed (even if it does not meet the criteria for partial regression) will be eligible to receive additional cycles of treatment using the autologous irradiated B7-

transfected cancer cells providing that they experienced no severe toxicity with the first vaccination. Patients may continue this treatment until they have evidence of progressive disease.

Initiation of work on Task 3 is dependent on the development of a FDA-approved, replication-defective, recombinant adenovirus bearing a human B7 cDNA (Ad.hB7) for use in human clinical trials. Our subcontractors at the University of Pennsylvania, Dr. James Wilson and Dr. Stephen Eck, have made significant progress toward this goal and anticipate that breast cancer patients will start accruing onto the Phase I trial in 1997. The following data summarizes their preclinical experience.

Ad.hB7-1:
in vitro gene transfer - Ad.hB7-1 transduces a variety of mammary and melanoma cell lines in vitro to express hB7-1. Using antibody staining and flow cytometry, at an MOI of 10, about 32% of WM9 human melanoma cells express hB7-1 three days after transduction; at an MOI of 100, nearly 100% of WM9 cells express hB7-1 three days after transduction..

in vivo gene transfer - Ad.hB7-1 was injected into WM9 human melanoma tumors established in the flank of SCID mice. Flow cytometric analysis of single cell suspensions made from the injected tumors revealed that 26%, 19% and 32% of tumor cells expressed hB7-1 at 7, 14 and 21 days after injection, sequentially.

Cells transduced with Ad.hB7-1 deliver a co-stimulatory signal to human T cells - Ad.hB7-1-transduced or Ad.lacZ-transduced WM9 human melanoma cells (89%+ for hB7-1 and 94%+ for lacZ, respectively, at three days; WM9 cells constitutively express MHC class I and II antigens) were treated with mitomycin C and cocultured with purified, allogeneic human peripheral blood T cells. T cell proliferation stimulation indices calculated on the basis of ^{3}H-thymidine incorporation by the T cells indicated that there was a 33-fold greater stimulation of proliferation by Ad.hB7-1-transduced WM9 cells than by Ad.lacZ-transduced WM9 cell and a 9-fold greater stimulation of proliferation by Ad.hB7-1-transduced WM9 cells than by untransduced WM9 cells. Similar results were obtained with Ad.hB7-1-transduced chinese hamster ovary (CHO) cells and WM793 human melanoma cells.

Ad.mB7-1:

in vitro gene transfer - Ad.mB7-1 transduces murine mammary and melanoma cell lines in vitro to express mB7-1 by flow cytometry. At an MOI of 1000, nearly 100% of K1735 murine melanoma cells express mB7-1 three days after transduction.

in vivo gene transfer - Ad.mB7-1 injected into subcutaneous K1735 tumors established in immunocompetent C3H/HeN mice resulted in tumor cell expression of mB7-1 determined

by flow cytometry and immunohistochemical staining on days 3 and 7 but not on day 15. Extinction of expression is presumably due to immunological elimination of cells expressing adenovirus vector antigens, as has been described before.

Efficacy of mB7-1 delivered by Ad.mB7-1 in inducing tumor rejection - K1735 murine melanoma cells were transduced with Ad.mB7-1 or Ad.lacZ in vitro at an MOI of 1000. After three days, 10^6 transduced or parental K1735 cells were injected into C3H/HeN mice. By 8 weeks, all 10 mice injected with parental K1735 cells developed progressive tumors, while 7/10 and 0/10 mice injected with Ad.lacZ- and Ad.mB7-1-transduced cells, respectively, developed progressive tumors. Mice injected with parental K1735 cells developed tumors at a median of 22 days, while mice injected with Ad.lacZ-transduced cells developed tumors at a median of 36 days. When surviving mice were rechallenged with parental K1735 cells 8 weeks after initial challenge, 3/3 and 3/10 of the mice that had survived their challenge of Ad.lacZ- and Ad.mB7-1-transduced cells, respectively, developed progressive tumors (all 10 naive mice injected at the time of rechallenge developed progressive tumors). The results obtained with Ad.mB7-1-transduced K1735 cells are comparable to those achieved using retrovirus-transduced, B7-1+ K1735 cells (including the relatively low frequency of protective immunity at 8-12 weeks in mice that previously rejected B7-1+ K1735 cells). Efforts to assess the efficacy of in vivo Ad.mB7-1-transduced K1735 tumor cells (Ad.mB7-1 injection intratumorally) have been unsuccessful. In part, this may be due to the fact that treatment cannot begin until the subcutaneous tumors reach a size that can be injected - about 3-4 mm in diameter. At this point, the rate of unperturbed K1735 tumor growth is such that C3H/HeN mice live a median of 8-9 additional days before they die of tumor or require euthanasia which may be insufficient time for effective host immunization and tumor control.

Ad.mB7-1 efficiently transduces SCK mammary carcinoma cells in vitro to express mB7-1. Injection of 2.5×10^4 live Ad.lacZ- or Ad.mB7-1-transduced SCK cells into A/J mice resulted in 9/10 mice in both groups developing tumors, while 6/10 mice given retrovirus-transduced mB7-1+ SCK cells developed tumors. An adverse effect on outcome of adenovirus transduction itself was excluded by the fact that a similar fraction (6/10) of mice developed tumors that had been given retrovirus-transduced mB7-1+ SCK cells supertransduced with Ad.lacZ. A potential explanation for the lack of protection by Ad.mB7-1-transduced SCK cells may come from the constraints of working with this aggressive tumor model. We routinely inject 2.5×10^4 live SCK cells after which tumors usually appear in 6-8 days and the mice are dead in 13-15 days. When we introduce 2.5×10^4 live SCK cells made B7-1+ by stable retrovirus transduction, all progeny SCK cells express B7-1, the number of B7-1+ tumor cells expands and the effective immunization dose increases. In contrast, when we introduce 2.5×10^4 live SCK cells made B7-1+ by Ad.mB7-1 transduction, assuming one viral genome/transduced cell, only half of the progeny of B7-1+ SCK cells will express B7-1, the number of B7-1+ tumor cells never increases above 2.5×10^4 and the effective immunization dose remains constant and low. Injecting more live SCK cells (e.g. 10^5 or 10^6 cells) is not a solution because these larger

inocula accelerate an already rapid disease course (death in 10-12 days) which we believe is inadequate to permit tumor immunization and immunological tumor rejection. Attempts to demonstrate the enhanced immunogenicity of Ad.mB7-1-transduced SCK cells by vaccination with 10^6 irradiated cells also have not succeeded, in part due to the enhanced immunogenicity of irradiated Ad.lacZ-transduced SCK tumor cells which may be preventing us from seeing an added benefit from vaccinating with mB7-1+, Ad-transduced cells.

The third round of plaque purification will be completed in the middle of May, 1997 at the Institute for Human Gene Therapy University of Pennsylvania. The FDA has indicated that the only toxicity study that will be necessary will be a study of Ad.mB7-1 in mice. It is anticipated that these studies will be completed by the end of August, 1997. The IND will be prepared and submitted by the Institute for Human Gene Therapy during August/September of 1997 and FDA approval is hoped for by October, 1997 and patient accrual onto the clinical trial by November or December, 1997.

Task 4

Task 4 is to perform in vitro and in vivo immunologic monitoring studies on patients enrolled on the clinical trial to assess the development of an anti-tumor immune response. Work on task 4 will begin as soon as patients begin treatment on the clinical trial estimated to start in the last quarter of 1997.

Additional animal experiments

Based on the recommendation of the group that reviewed the original grant, an animal model of breast cancer was developed to test the gene therapy proposed in the grant, before beginning the clinical trial in patients.

Dr. Fred Chang's laboratory at the University of Michigan studied a mammary carcinoma, MT-7 in Balb/c mice. The attached manuscript provides additional details and has been accepted for publication in *Cancer Gene Therapy*. MT-7 is a cultured tumor cell line derived from a dimethylbenzanthracene-induced mammary carcinoma in the Balb/c host. A subline, MT-901, was derived from an early in vitro passage of cultured MT-7 tumor inoculated subcutaneously. MT-901 cells were determined to be weakly immunogenic in traditional immunization and challenge experiments. MT-901 cells that were genetically modified to express the co-stimulatory molecule B7-1 failed to generate tumors in two out of five mice that were inoculated subcutaneously whereas five out of five mice had tumor growth when inoculated with the wild-type MT-901 tumor cells. MT-901 cells that were genetically modified to secrete GM-CSF also grew less well than wild-type MT-901 with no tumor growth in two out of five mice inoculated with a low GM-CSF secreting clone.

In immunization and challenge experiments neither genetic modification resulted in superior protection against a subsequent tumor challenge compared to wild-type tumor alone. In separate experiments, MT-901 cells that were genetically modified to secrete IL-12, initially grew, but then were rejected in all five mice that were inoculated. However, subsequent challenge of these mice with wild-type tumor cells resulted in tumor growth in all the animals.

The genetically modified MT-901 tumor cells were then tested for their ability to sensitize tumor draining lymph node cells (TDLN) for adoptive immunotherapy. The TDLN were harvested nine days after the inoculation of tumor cells and they were activated and expanded with anti-CD3 plus IL-2 in vitro and adoptively transferred into mice bearing three day established MT-901 pulmonary metastases. The B7-1 expressing clone induced pre-effector cells better than wild-type tumor in one of two experiments. The low GM-CSF secreting clone was no different than wild-type tumor, but the high GM-CSF secreting clone was significantly better than wild-type tumor in the induction of tumor reactive TDLN. In a similar but separate experiment, the IL-12 transfected clone failed to elicit pre-effector TDLN cells differently from wild-type tumor.

Subsequent experiments designed to determine the type of effector cell that mediated tumor regression with the successful GM-CSF secreting clone indicated that $CD4^+$ cells mediate the effect. The CD4 mediated cytotoxicity appears to be related to *fas* ligation because the addition of *fas* fusion protein inhibited the in vitro cytotoxicity of these $CD4^+$ cells.

Dr. Chang's lab is currently exploring alternative approaches that may take advantage of B7-1 transgene expression, for example, by combining it with the co-expression of transgenes encoding for the cytokines GM-CSF or IL-12.

<u>Additional experiments</u>

Because of the delay involved in obtaining the adenoviral vector from our subcontractor, Dr. Ethier's lab conducted experiments to test the suitability of other vectors for possible use in the clinical trial. Lipofection with a plasmid vector and delivery by a gene gun failed to get the LacZ gene into more than a small percentage of cultured human breast cancer cell lines and was therefore abandoned as a possible alternative strategy.

III. Conclusions

The progress reported in this summary of year two of this grant indicates that the major objectives of Task 1 and Task 2 of the Statement of Work have been achieved, paving the way for the clinical trial that will begin in 1997. Dr. Ethier's laboratory has clearly demonstrated the feasibility of short-term culture of human breast cancer cells necessary to perform a vaccination trial where autologous tumor cells are transduced with an adenoviral vector ex vivo. Dr. Ethier's laboratory has also documented the success of the adenoviral expression vector, Ad.hB7, in both breast cancer cell lines and primary cultures of human breast cancer cells with more than 90% of the cells expressing B7-1 on their surface for at least one month. These accomplishments demonstrate that the experimental approach that will be utilized in the clinical trial, i.e. ex vivo incubation of autologous breast cancer cells with the human B7-1 adenoviral expression vector, Ad.hB7, is technically feasible and that these transduced cells should express B7-1 sufficiently long in vivo for vaccination purposes.

Experiments conducted in two different mouse mammary carcinoma models support the hypothesis that B-7 expression enhances the immune recognition of tumor cells. Both models also point to additional cytokines (i.e. IL-12 or GM-CSF) that may improve the immune mediated regression of tumors in combination with B7-1 expression and will be studied further in the next year. After the safety of B7-1 transduced tumor cells is demonstrated in patients, the addition of these cytokines can be contemplated.

IV. References

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Table 1.

Molecular Characteristics of "SUM" HBC Cell Lines

Cell Line	Oncogene Amp.*	EGFR	erbB-2	erbB-3	erbB-4	p53 (IHC)
SUM-44PE	FGFR-1	–	+	+	+	+ (c)
SUM-52PE	FGFR-1 & 2	–	+	+	+	+ (c)
SUM-16LN	EGFR	++++	NE	NE	NE	NE
SUM-102PT	none	+++	+	+	–	–
SUM-149PT	none	+++	+	+	–	+ (n)
SUM-159PT	none	+	+	–	+	+ (n)
SUM-1315MO2	NE	+	+	–	+	+ (n)
SUM-185PE	erbB-2 (?)	+	+++	NE	NE	NE
SUM-190PT	erbB-2 (?)	+	+++	NE	NE	+ (n)
SUM-206CWN	NE	+	++	NE	NE	+ (n)
SUM-224PE	NE	NE	NE	NE	NE	NE
SUM-225CWN	NE	NE	NE	NE	NE	NE
SUM-229PE	NE	NE	NE	NE	NE	NE

*Oncogenes examined by Southern blot: erbB-2, c-myc, Prod-1, FGFR-1, 2, 4, EGFR
NE - not examined

Note that in the table, cell lines designated PT are from primary tumors, PE are from pleural effusions, LN are from metastatic lymph nodes, and CWN are from chest wall nodules.

Table 2

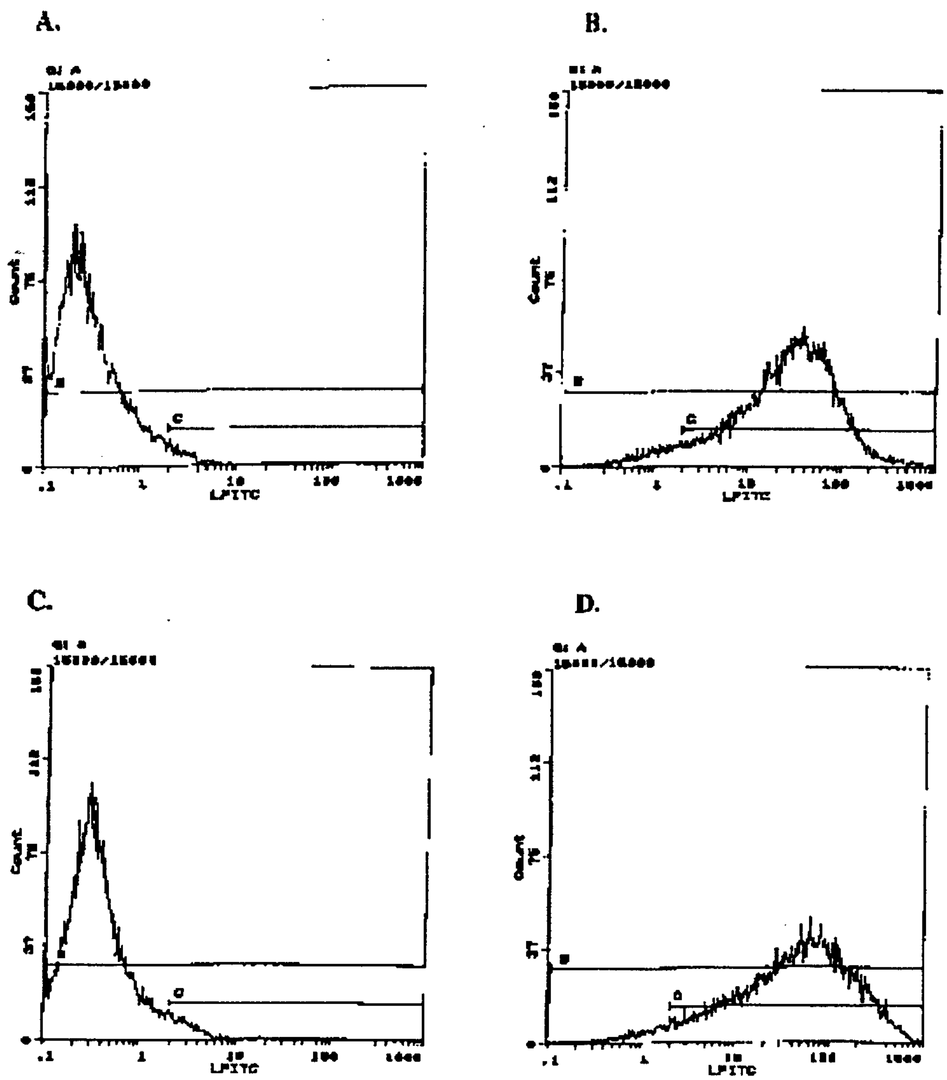
B7 Expression in Human Breast Cancer Cell Lines

Multiplicity of infection (pfu/cell)

Cell Line	0	10^4	10^3
Sum-102	7.9	98.4	ND
	3.3	84.7	ND
Sum-149	4.3	92.8	ND
	5.1	75.6	38.9
Sum-159	3.0	92.9	ND
	12.4	97.9	ND
Sum-44	8.7	96.9	ND
	2.2	58.3	34.0
Sum-52	6.6	79.2	75.1

[a] percent B7-1 positive cells by flow cytometry

Figure 1.



FACS analysis of B7.1 expression in two human breast cancer cell lines. Panels A and B show data from SUM-149 cells and panels C and D show data from SUM-159. Left hand panels show background fluorescence of control cells, and panels B and D show B7.1 specific fluorescence of AdB7.1 infected cells.

V. Appendices

Original research project proposal.

Letter to Danny Laspe.

Subcontract with Earle A. Chiles Research Institute.

Attached manuscript (in press, *Cancer Gene Therapy*): Immune Responsiveness to a Murine Mammary Carcinoma Modified to Express B7-1, IL-12 or GM-CSF. E. Aruga, A. Aruga, M. Arca, W. Lee, N. Yang, J.W. Smith, A. Chang. (1996)